MYCOTAXON

ISSN (print) 0093-4666 (online) 2154-8889 Mycotaxon, Ltd. ©2017

January–March 2017—Volume 132, pp. 99–106

http://dx.doi.org/10.5248/132.99

Two new records of puffballs in Thailand

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Abstract—*Calvatia holothurioides* and *Morganella puiggarii,* two puffballs in the *Agaricaceae,* are reported for the first time from Thailand. The specimens were identified based on morphological and ITS sequence analyses. Descriptions and illustrations are provided.

Key words—*Agaricales*, basidiomycete, phylogeny, taxonomy

Introduction

Puffballs in the genera *Bovista*, *Bovistella*, *Calvatia*, *Lycoperdon*, *Morganella*, *Tulostoma*, *Utraria*, and *Vascellum*, formerly classified as *Lycoperdaceae*, are now included in *Agaricaceae* (Kirk et al. 2008). Recent molecular phylogenetic studies have proposed a widening of *Lycoperdon* to incorporate traditional genera such as *Bovistella*, *Morganella*, *Utraria*, and *Vascellum* (Larsson & Jeppson 2008, Gube 2009). During a taxonomic survey of puffballs in northern Thailand, we collected specimens that corresponded to *Calvatia holothurioides* and *Morganella puiggarii,* species previously unknown in the country. Here we present the morphological characters and ITS sequence analyses of the Thai specimens.

Materials & methods

Morphology

Basidiomes were collected from Doi Suthep-Pui and Doi Inthanon National Parks, Chiang Mai Province, Thailand in 2013, and wrapped in aluminum foil or

placed in plastic specimen boxes for transport to the laboratory, where notes on macromorphological features and photographs were taken within 24 h. Color names and codes follow Kornerup & Wanscher (1978). The specimens were dried at 40–45 °C and deposited in the herbarium of the Research Laboratory for Excellence in Sustainable Development of Biological Resources, Faculty of Science, Chiang Mai University, Thailand (SDBR-CMU). Tissues from dried specimens were rehydrated in 95% ethanol followed by distilled water, 3% KOH, or Melzer's reagent for microscopical examination. Dimensions are based on at least 50 measurements of each anatomical structure. Scanning electron images of basidiospores were captured with a JEOL JSM-5910LV electron microscope.

DNA extraction & amplification

DNA was extracted from fresh specimens using a Favorgen® Genomic DNA Extraction Mini Kit. The internal transcribed spacer (ITS) region of ribosomal RNA gene was amplified by the polymerase chain reaction (PCR) with ITS4 and ITS5 primers under the following thermal conditions: 94 °C for 2 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min; and finally 72 °C for 10 min. PCR products were checked on 1% agarose gels stained with ethidium bromide under UV light and purified using NucleoSpin® Gel and PCR Clean-up Kit following the manufacturer's protocol. The purified PCR products were directly sequenced. Sanger sequencing was performed by 1ST Base Company (Kembangan, Malaysia) using the same ITS4 and ITS5 PCR primers. The new sequences were used to query GenBank database via BLAST (http://blast.ddbj.nig.ac.jp/top-e.html).

Phylogenetic analysis

A multiple alignment subroutine in Clustal X (Thompson et al. 1997) and phylogenetic tree was generated using the PAUP beta 10 vers. 4.0 (Swofford 2002). In maximum parsimony analysis, all characters were equally weighted and gaps were treated as missing data. Heuristic searches were performed with 100 random-addition sequence replicates and tree-bisection-reconnection branch swapping. Bootstrap analysis (Felsenstein 1985) was conducted with 1000 replicates using the same settings as above.

Taxonomy

Calvatia holothurioides Rebriev, Mikol. Fitopatol. 47: 21 (2013). Fig. 1

Basidiomes pyriform, turbinate to broadly excipuliform, 3–6 cm diam and 4–6 cm high. Exoperidium two-layered, brownish orange (6C6) to brown (7E4), slightly wrinkled, formed by hyaline, septate and branched hyphae, 4–7 μm wide, intermixed with hyaline, thin-walled vesicles 12–20 μm diam, with an underlying pseudoparenchymatous layer. Endoperidium light brown (7D6), formed by septate, thin-walled hyphae, 3–5 μm diam. Gleba cottony, yellowish white (4A2) when young, light brown (7D6) when mature. Subgleba light brown (7D6), composed of compacted cells. Paracapillitium

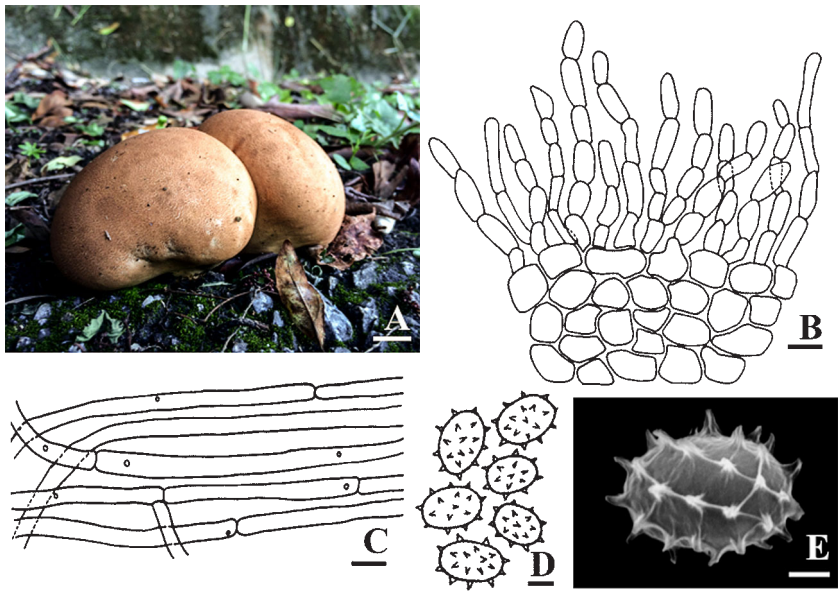

Fig. 1. *Calvatia holothurioides* (SDBR-CMU-NK0152). A: basidiomes; B: exoperidial layer; C: paracapillitial hyphae; D: basidiospores (LM); E: basidiospore (SEM). Scale bars: A = 10 mm, B = 10 µm, C = 5 µm, D = 2 µm, E = 1 µm.

thin-walled, septate, branched, hyaline, 3–5 µm diam. Pores roundish, ≤1.5 µm diam. Basidiospores ellipsoid to elongate, 3–5.5 × 2–3 µm (without ornamentation), Q = 1.5–1.8; hyaline in water and KOH, unchanging in Melzer's reagent; echinate, shortly pedicellate. Spines 0.2–0.5 µm long.

Specimen examined—**THAILAND**, **Chiang Mai Province**, **Muang District**, Doi Suthep-Pui National Park, 18°47′34″N 98°55′49″E, elevation 746 m, on soil in deciduous forest, 1 June 2013, Suwannarach N. & Kumla J. (SDBR-CMU-NK0152, GenBank KX064242).

Morganella puiggarii (Speg.) Kreisel & Dring, Feddes Repert 74: 116 (1967). Fig. 2

Basidiomes globose to subglobose, 0.6–2 cm diam. and 0.9–2.3 cm high, with whitish rhizomorphs attached at the base. Exoperidium white (3A1) to yellowish white (3A2) when fresh, dark brown (6F6) when dried, covered by minute conical tubercles, composed of chains of globose cells, 8–22 × 12–28 µm diam. Endoperidium soft, white (3A1). Gleba when young cottony and white (3A1), when mature powdery and dark brown (7F7). Pseudocolumella present. Subgleba orange-white (5A2), composed of compacted cells.

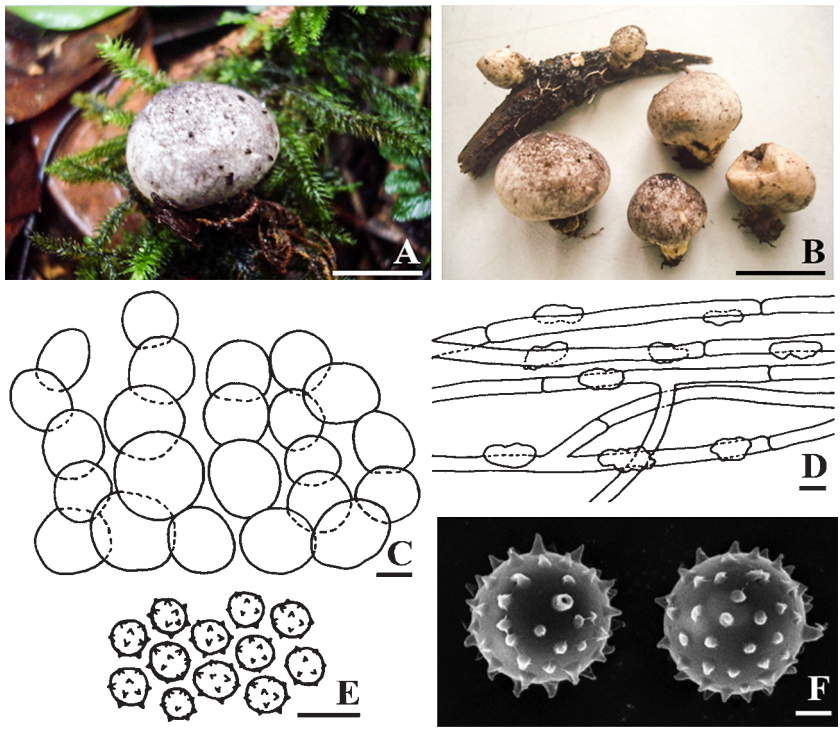

Fig. 2. *Morganella puiggarii* (SDBR-CMU-NK0141). A, B: basidiomes; C: exoperidial elements; D: paracapillitial hyphae; E: basidiospores (LM); F: basidiospores (SEM). Scale bars: A, B = 10 mm, C = 10 µm, D = 5 µm, E = 5 µm, F = 5 µm.

Paracapillitium thin-walled, 1–2.5 µm diam., septate, branched, hyaline, covered by an amorphous hyaline incrustation. Basidiospores globose, 3–4 µm diam. (without ornamentation), Q = 1–1.05; pale orange (5A3) in water and KOH, unchanging in Melzer's reagent; echinate, shortly pedicellate. Spines ≤0.5 µm long.

Specimen examined—**THAILAND, Chiang Mai Province, Chom Thong District**, Doi Inthanon National Park, 18°34′16″N 98°28′56″E, elevation 235 m, on rotting wood in mixed evergreen hill forest, 12 July 2013, Suwannarach N., Kumla J. & Lumyong S. (SDBR-CMU-NK0152, GenBank KX064241).

ITS sequence and phylogenetic analysis

The ITS sequences of *C. holothurioides* SDBR-CMU-NK0152 (659 bp) and *M. puiggarii* SDBR-CMU-NK0141 (699 bp) were deposited in GenBank. The

similarity search revealed a 98% similarity between SDBR-CMU-NK0152 and *C. holothurioides* JQ734547 and KJ909662 and a 96% similarity between SDBR-CMU-NK0141 and *M. purpurascens* (Berk. & M.A. Curtis) Kreisel & Dring KC414581.

In the phylogenetic analysis, the aligned dataset of 30 ITS sequences comprised 745 characters, of which 454 characters were constant, 76 variable characters were parsimony uninformative, and 215 characters were parsimony

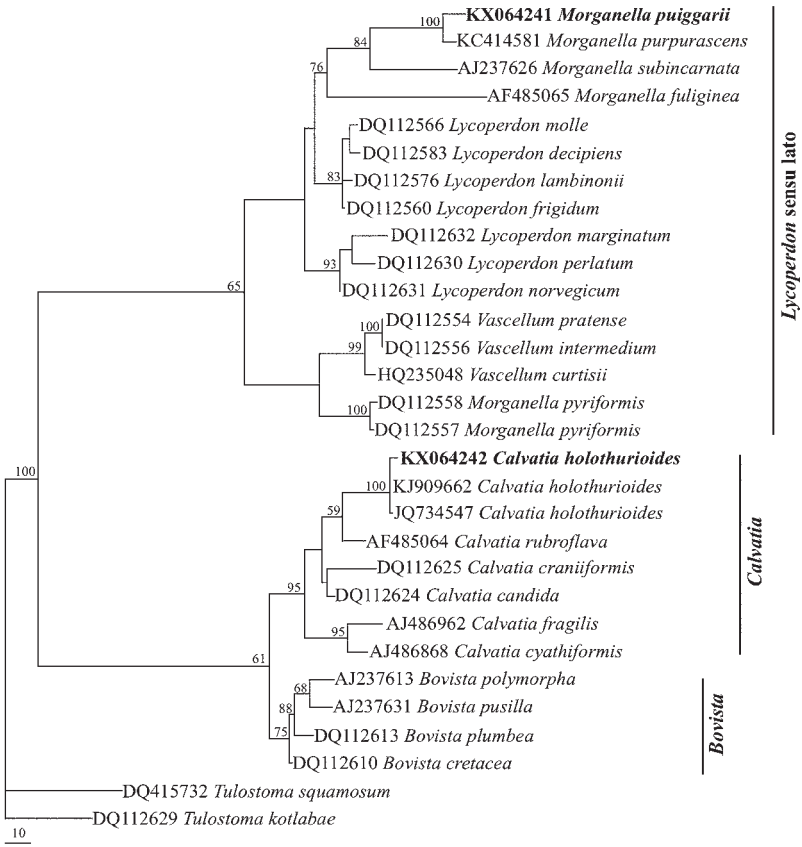

Fig. 3. One of 62 maximum parsimonious trees of 30 ITS sequences. *Tulostoma kotlabae* and *T. squamosum* were used as the outgroup. Numbers above branches show bootstrap supports ≥50%; scale bar represents ten substitutions per nucleotide position. The sequences obtained in this study are in bold.

informative. Heuristic searches produced a length of 667 steps, CI = 0.609, RI = 0.686, RC = 0.417 and HI = 0.391. One of 62 maximum parsimonious trees (Fig. 3) clustered our *C. holothurioides* ITS sequence with *C. holothurioides* sequences from GenBank within the *Calvatia* clade. Our sequence of *Morganella puiggarii* was assigned to the *Lycoperdon* sensu lato clade and formed a sister taxon to *M. purpurascens* with 100% bootstrap support.

Discussion

Calonge et al. (2003) assigned *C. holothurioides* to "*Calvatia* sect. *Sporocristata*" based on its echinulate and ellipsoidal spores. This invalid section contained only two species, *C. oblongispora* V.L. Suárez et al. and *C. sporocristata* Calonge (Calonge et al. 2003, Suárez et al. 2009). *Calvatia holothurioides* clearly differs from *C. oblongispora* and *C. sporocristata* by its pyriform to broadly excipuliform basidiomes and smaller spores (Calonge et al. 2003; Suárez et al. 2009; Rebriev 2013). *Calvatia holothurioides* has been reported previously from Vietnam (Rebriev 2013).

Morganella puiggarii differs from *M. arenicola* Alfredo & Baseia, *M. albostipitata* Baseia & Alfredo, *M. benjaminii* (Rick) Cortez et al., *M. compacta* (G. Cunn.) Kreisel & Dring, *M. fuliginea* (Berk. & M.A. Curtis) Kreisel & Dring, *M. nuda* Alfredo & Baseia, *M. stercoraria* P. Ponce de León, *M. rimosa* Baseia & Alfredo, *M. sulcatostoma* C.R. Alves & Cortez, and *M. velutina* (Berk. & M.A. Curtis) Kreisel & Dring by its tuberculate exoperidium (Ponce de León 1971; Cortez et al. 2007; Alfredo et al. 2012, 2014; Alves & Cortez 2013, 2014). The presence of an eucapillitium in *M. pyriformis* (Schaeff.) Kreisel & D. Krüger clearly separates this species from *M. puiggarii*. The paracapillitium of *M. samoensis* (Bres. & Pat) P. Ponce de León and *M. subincarnata* (Peck) Kreisel & Dring is wider than of *M. puiggarii* (Ponce de León 1971). *Morganella puiggarii* forms a sister taxon to *M. purpurascens*, the latter having a pitted endoperidium (Ponce de León 1971). Moreover, *M. puiggarii* was previously known from Brazil and Paraguay (Ponce de León 1971).

The combination of morphological and molecular features supports *C. holothurioides* and *M. puiggarii* as new records for Thailand. Prior to this study, there have been no reports of *Calvatia* "sect. *Sporocristata*" and only four *Morganella* species (*M. compacta*, *M. fuliginea*, *M. purpurascens* and *M. pyriformis*) recorded from the country (Phanichapol 1968, Ellingsen 1982, Kasuya et al. 2006, Chandrasrikul et al. 2011, Kumla et al. 2014).

Acknowledgements

This work was supported by grants from Chiang Mai University and Thailand Research Fund, Research-Team Association Grant (RTA5880006). We also thank Dr. Eric H.C. McKenzie and Dr. Steven L. Stephenson for their helpful comments and careful review of this article.

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